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This project aims to devise a strategy for maintaining butyric acidemia in mice over a 24 hour period of time, so as to achieve a nearly complete inhibition of histone deacetylase activity. The basic approach is to use butyrylglycerides as a prodrug to butyrate and MCPA as an inhibitor of butyrate metabolism (MCPA). We monitor for both vital signs and blood chemistries to assure survival during the prolonged metabolic acidosis. Our hypothesis is that such a scheme will allow maintenance of butyrate blood levels between 1 and 5 mM, and that the mice will develop hyperacetylation of chromatin core histones sufficient to cause extensive cytolysis in xenografted tumors with known sensitivity to butyrate-induced apoptosis.

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John A. Bain 26 NOV '99

PI - Signature Date

Butyrate therapy for poorly differentiated breast cancer

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Table of Contents

John A. McBain, PhD
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IDEA award
Progress Report
November, 1999

Butyrate therapy of poorly differentiated breast cancer

Introduction

This project aims to devise a strategy for maintaining butyric acidemia in mice over a 24 hour period of time, so as to achieve a nearly complete inhibition of histone deacetylase activity. The basic approach is to use butyrylglycerides as a prodrug to butyrate, and methylenecyclopropylacetate (MCPA[1]) as an inhibitor of butyrate catabolism. We monitor for both vital signs and blood chemistries to assure survival during the prolonged metabolic acidosis. Our hypothesis is that such a scheme will allow maintenance of blood butyrate concentrations at levels between 1 and 5 mM, and that each of the mice will develop hyperacetylation of chromatin core histones. Such hyperacetylation should be sufficient to cause extensive cytolysis in xenografted tumors with known sensitivity to butyrate-induced apoptosis.

The goals of this study remain:

1. Determine the pharmacokinetics of butyric acidemia, demonstrating the effectiveness of intraperitoneally-delivered butyrylglyceride in mice treated (or not) with MCPA.
2. Document core histone acetylation state of chromatin isolated from selected tissues, as an indicator of the inhibition of histone deacetylase, and thereby the bioavailability of butyrate within the target cells.
3. Document and attempt to correct untoward acute effects of MCPA and butyrate therapy. These effects are predicted to be hypothermia, hypoglycemia, metabolic acidosis, electrolyte disturbances and nausea.
4. Document untoward delayed or chronic effects of butyrate therapy, including bone marrow depression (as reflected in reductions in blood cell counts) and liver toxicity (e.g., fatty liver or necrosis).
5. Demonstrate antitumor effects of prolonged butyrate therapy, in relationship to hyperacetylation of tumor chromatin histones.

Body

As previously reported (1998 annual report), intraperitoneal tributyrin emulsion was able to provide butyric acidemia in excess of 5 mM for periods of up to one half hour in control mice, but up to 3 hr in MCPA pre-treated mice. Replicate determinations were highly variable, especially at later times after administration of emulsion, results that were reflected in variability of the depth of depression of consciousness and stimulation of respiration[2]. We attributed this variability to unfavorable characteristics of the oil-

[1] Abbreviations: MCPA, methylenecyclopropylacetate; SCAD, short chain acylCoA dehydrogenase; MCAD, medium chain acylCoA dehydrogenase; TPN, total parenteral nutrition; FEP, a teflon-like plastic; EVA, ethylenevinylacetate, an elastomeric plastic.

[2] Although we did not try to quantify these later effects in individual mice, consciousness appeared to be impaired in mice with blood concentrations of butyrate above ~3 mM, and respiratory stimulation increased

in-water butyric triglyceride emulsion. Our trials with intravenous sodium butyrate indicate that the half life of butyrate, starting from about 4 mM in plasma, is about 9 minutes. Although MCPA prolongs this halflife about 4-6 fold (we have samples of a more complete and higher quality experiment awaiting assay), this timeframe is still short enough for the results with i.p. administration to be dominated by variability in the distribution of the emulsion within the peritoneal cavity (rapid release by hydrolysis of widely-dispersed emulsion,as opposed to slow, steady release by hydrolysis at the periphery of larger pools of emulsion).

A second and third administration of tributyrin emulsion resulted in additional peaks of butyric acidemia, and exacerbations of constitutional symptoms of acidosis. As expected from the variable level of the residual/background butyric acid in these mice, the results for this second peak of butyric acidemia exhibited compounded variability. However, it was nonetheless clear that the duration of this second period of butyric acidemia was abbreviated, even in MCPA-pretreated mice. This was taken to represent recovery from MCPA-induced inhibition of butyrate metabolism, presumably by resynthesis of short-chain acylCoA dehydrogenase (SCAD), although other adaptive mechanisms, like increased renal clearance of butyrate could be responsible. In agreement with the first possibility, an additional MCPA treatment restored the response (increased butyric acidemia from the second administration of tributyrin). But when MCPA was given after butyric acidemia had been initiated, the degree of enhancement was less than if MCPA had been given to animals prior to the first tributyrin injection. This suggested that butyrate interfered with MCPA for uptake into the cell, binding to SCAD, or both. Use of MCPA to extend butyric acidemia over 20 hr will thus necessitate administration of this agent continuously at concentrations higher that that which is useful in a pretreatment sense, and increasing the chances of inhibition of alternative enzymes such as the medium chain acylCoA dehydrogenase.

The modifications to the approach that we are using include the following:

a. **Continuous Infusion via Intraperitoneal catheter** – We received IACUC approval for this modification to our approach. While we had suggested administering a benzodiazepine to all mice as a component of the infusate, to confer flaccid unconsciousness in the animals that were not unconscious due to butyric acid, thereby obviating the need to secure the catheter, the committee insisted instead that we obtain swivel sets for infusion irrespective of activity level. These devices allow freedom of movement for the animal during continuous administration of fluid. Considering the high cost of these devices relative to osmotic minipumps ($250 each as opposed to $20 each), one might ask why we do not use the later (we can afford neither, so this is a moot point). In fact, we wish to be able to modify and ascertain the infusion rates, at times increasing or decreasing the flow through one of the two lines that will converge prior to the swivel. The catheter (we have chosen a Microline crosslinked-EVA tubing, both for biocompatibility and chemical compatibility reasons) will run under the skin from the dorsal neck to the lateral peritoneum, where it will be left indwelling through the peritoneum. The wall of the catheter is cut to an open-end conical form, and held around a 22g hypodermic needle for insertion. This plastic is otherwise very soft, similar to silicone, and unlikely to puncture any hollow viscus. We have successfully tested this on a series of mice. We have obtained two 10 channel syringe pumps, one for a monobutyrin and MCPA

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over butyrate concentrations from 3 to 10 mM. Respiratory signs and the decrease in blood pH appeared to be relieved significantly by administration of bicarbonate and glucose.

solution (the output of which will be monitored and recorded on a computer interface over time), and another, for a glucose/sodium bicarbonate solution, which is modified to allow transitory increases of individual syringes. The later will be provided for hydration and relief of untoward effects of the SCFA therapy. These two lines will be joined at the swivel by means of high pressure fittings.

b. **Monobutyrin as an alternative prodrug** – Tributyrin, the choice of the NCI Division of Cancer Treatment as a butyrate pro-drug[3], has limited usefulness as a parenteral agent or as a slow-release reservoir of butyrate anion. A better approach to modeling cell culture experiments would be parenteral infusion of a water-soluble ester or salt. The use of the sodium salt would, we predict, risk hypernatremia, especially as sodium bicarbonate will likely be needed throughout the infusion. Monobutyrin has been given to dogs and rats at 1-4 g/kg/day (isocaloric replacement for glucose), providing weight gain and no reported untoward effects, as it was under investigation as an energy source as a carbon/energy source in a TPN formulation for human use.

   The later study prepared a crude monobutyrin by partial alkaline saponification of tributyrin, leading to a water-soluble mixture of monobutyrin, dibutyrin and presumably fatty acid and glycerol. Other workers have prepared monobutyrin by purchasing technical grade monobutyrin from Eastman Chemicals and distilling it under vacuum. Eastman Chemicals no longer exists except to provide products to Fisher Scientific, who can no longer obtain monobutyrin, leaving no alternative source. However, the need for vacuum distillation, and the analytical procedures required to verify its purity make it worthwhile to synthesize the agent in the laboratory (by simple esterification). We have thus obtained and setup the necessary equipment and chemicals needed for synthesis and purification of monobutyrin, and have begun to set up the analytical instrumentation.

   The approach for this synthesis is basically an anhydrous reaction of n-butyryl chloride with glycerol in chloroform/pyridine. We anticipate that monobutyrin will prove somewhat unstable, explaining why the only commercial preparations are technical grade, and necessitating preparation at the time of use or storage under strictly anhydrous conditions. The product will be analyzed for carboxylic acid content, proportion of dibutyrin and longer chain glycerides, and presence of non-volatile material. We will determine the rate of accumulation of butyric acid, as an indicator of hydrolysis, in an anhydrous neat form and in a 10% aqueous solution, with or without tris HCl buffer. We will also test for the monoglyceride as a circulating species during infusion.

c. **MCPA as a continuous infusion as well as bolus pretreatment** – The short effective period of MCPA in treated mice indicates that mice will require continuous infusions of MCPA after the bolus pretreatment, and that the interference of circulating butyrate with supplemental MCPA will necessitate detailed testing of monobutyrin/MCPA infusion conditions. Our work has demonstrated that single dose MCPA, with or without tributyrin at +30 minutes, is well tolerated even at doses 3 times that of our standard 15 mg/kg pretreatment dose. Earlier work with hypoglycin, the amino acid precursor of MCPA[4], demonstrated hypoglycemia,

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[3] Several preclinical and phase 1 clinical trials have been carried out to determine the maximal tolerated dose and pharmacokinetics of this agent, generally as an oral formulation.

[4] Hypoglycin is the toxic constituent present in Ackee nut, responsible for Jamaican vomiting sickness which befalls persons who ingest unripe breadfruit (Ackee nuts). Upon deamination, hypoglycin yields MCPA, which as a CoA ester acts as a suicide inhibitor of dehydrogenases SCAD (short chain acylCoA dehydrogenase), MCAD (medium chain), IVAD (isovalerylCoA dehydrogenase). Because of the general depression in fat metabolism, both from inhibition of dehydrogenases acting upon acyl groups less than 10-

hypothermia, acidosis and delayed effects such as thymic involution and liver damage. Since we control for hypothermia (which has been shown to exacerbate the hypoglycemia), and ameliorate the acidosis somewhat using bicarbonate, our mice are somewhat better supported that mice in earlier, toxicology experiments. However, we cannot predict the outcome of experiments with continuous infusion with the higher doses, especially as the butyrate may protect the SCAD from MCPA inactivation, but is unlikely to protect MCAD from inactivation, and MCAD deficiency is a more clinically significant genetic deficiency disease than is SCAD deficiency. We will thus examine the blood of such mice for longer chain fatty acids as possible evidence for accumulation of MCAD substrates such as octanoic acid.

d. **Environmental control** – Because of the propensity of MCPA-treated mice to develop hypothermia, we have constructed a temperature controlled (33-36°C across the platform) ventilated multichambered incubator for long-term infusion, physiological monitoring and blood sampling of mice. The reproducibility and stability of chamber temperatures, and rectal temperatures of the mice, indicate that device is effective.

e. **Rapid, low sample volume analysis of blood pH and butyrate** - Our work documenting pH changes during butyric acidemia and after administration of sodium bicarbonate to such mice pointed out a deficiency in the ability to gauge the effects of the test agents and remedial interventions on individual mice. Because our pH and pCO_2 apparatus had not been miniaturized for samples under 50-100 µl, we could not monitor the condition of the mice without killing them (or observing them for respiratory activity). We have designed and begun construction of a device to measure pH and pCO_2 on smaller samples (30-50 µl), conceivably allowing serial sampling of living mice, i.e., from tail vein or intraorbital venous plexus. After reading the electrode responses, the diluted blood will be deproteinized and used for determination of glucose.

f. **Physiological monitoring** – Besides the need for blood chemistry determinations during the course of infusion (which would be best satisfied by implantable electrodes, but that is beyond our financial means), we believe that the health of the mice during prolonged MCPA/butyrate infusion will be better protected if we monitor and respond to changes in respiratory activity, conscious movement and core body temperature. We have obtained a MacLab analog/digital converter, biopreamp and several transducers (including a bellyband for diaphramatic activity related to respiration depth and rate). The rapid response of respiratory activity to blood pH, pCO_2 and organic acids may allow us to better gauge and record the response to bicarbonate and changes in butyrate infusion rate, while core body temperature may provide an indication of the severity of hypoglyemia.

g. **Core histone acetylation ratio** - Most of the phenomena associated with growth inhibition or apoptosis induction require over 12-16 hr of treatment with millimolar concentrations of butyrate to be evident or significantly increased. This corresponds to the kinetics and dose response relationships of cells to butyrate as evident in hyperacetylation of bulk histones, i.e., the species of histones H2a, 2b, 3 and 4 that are evident on Coomassie blue-stained electropherograms. Such changes are rapidly (15 min) reversed upon reduction of butyrate concentrations. Because the problems mentioned above have precluded maintenance of millimolar butyrate concentrations for beyond 2-3 hr, we believe that this determination would be futile at the present time, but will be an important determination once we have a continuous treatment regimen. At such a time, an important question is whether

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12 carbons, and depletion of CoA, general energy metabolism becomes deficient. This leads to excessive, life-threatening consumption of glucose, and hypothermia.

inhibition of intracellular metabolism will reduce the plasma butyrate concentration necessary for significant histone hyperacetylation. This is of particular interest in this project because the intracellular butyrate concentration is likely to be closer to the absolute plasma butyrate level in MCPA-treated animals than in control animals. It has often been assumed that the reason why 5 mM butyrate is required for biological effect, while the deacetylase is half inhibited at 150 μM, is because conversion of butyrate to butyrylCoA (and subsequent consumption of butyrylCoA) depletes cells of non-esterified butyrate. We predict that submillimolar plasma concentrations of butyrate might achieve significant deacetylase-inhibitory intracellular concentrations, and hope to see this.

h. **Bone marrow and liver cell survival and growth** – As with the effects of butyrate on the histone acetylation pattern, we do not anticipate finding any change in cell or tissue architecture or cell survival due to the short exposure (2-3 hr) to millimolar butyrate concentrations. Several mice that went through this experience were allowed to recover and then followed for up to 4 months thereafter, and out of more than 6 mice, no subacute or chronic changes were notable. Again, this type of determination will be an important question to follow in mice receiving continuous exposure to various concentrations of butyric acid and MCPA.

Conclusion and prospects

This project has evolved into one which focusses increasingly on butyrate as a determinant of life-threatening acidosis, on miniaturization of procedures for continuous infusion and physiological monitoring of mice, and of dealing with pharmaceutically difficult agents, including an inhibitor of metabolism that is inhibited in its action by the very substrate that it is designed to protect. In spite of these challenges (and a complete lack of funds), we are committed to carrying the endeavor through. We resolve to demonstrate the relative ability of butyrate to serve as a therapeutic inhibitor of histone deacetylase, hopefully before the establishment of some of the 5 or more fungal or synthetic inhibitors that are being aggressively pursued by their patent holders.